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## Evaluation of phthalazinone phosphodiesterase inhibitors with improved activity and selectivity against *Trypanosoma cruzi*

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**Background:** Chagas' disease, caused by the protozoan parasite *Trypanosoma cruzi*, needs urgent alternative therapeutic options as the treatments currently available display severe limitations, mainly related to efficacy and toxicity.

**Objectives:** As phosphodiesterases (PDEs) have been claimed as novel targets against *T. cruzi*, our aim was to evaluate the biological aspects of 12 new phthalazinone PDE inhibitors against different *T. cruzi* strains and parasite forms relevant for human infection.

**Methods:** *In vitro* trypanocidal activity of the inhibitors was assessed alone and in combination with benznidazole. Their effects on parasite ultrastructural and cAMP levels were determined. PDE mRNA levels from the different *T. cruzi* forms were measured by quantitative reverse transcription PCR.

**Results:** Five TcrPDEs were found to be expressed in all parasite stages. Four compounds displayed strong effects against intracellular amastigotes. Against bloodstream trypomastigotes (BTs), three were at least as potent as benznidazole. *In vitro* combination therapy with one of the most active inhibitors on both parasite forms (NPD-040) plus benznidazole demonstrated a quite synergistic profile ( $x\Sigma$  FICI = 0.58) against intracellular amastigotes but no interaction ( $x\Sigma$  FICI = 1.27) when BTs were assayed. BTs treated with NPD-040 presented disrupted Golgi apparatus, a swollen flagellar pocket and signs of autophagy. cAMP measurements of untreated parasites showed that amastigotes have higher ability to efflux this second messenger than BTs. NPD-001 and NPD-040 increase the intracellular cAMP content in both BTs and amastigotes, which is also released into the extracellular milieu.

**Conclusions:** The findings demonstrate the potential of PDE inhibitors as anti-*T. cruzi* drug candidates.

## Introduction

About 8 million people are infected with the protozoan parasite *Trypanosoma cruzi*, the aetiological agent of Chagas' disease, which is the main cause of infectious cardiomyopathy in Latin America.<sup>1</sup> Introduced ~50 years ago into clinical practice, the nitroheterocyclic compounds benznidazole and nifurtimox are still the only therapeutic options, despite serious concerns such as: limited efficacy, especially in the chronic phase; the occurrence of naturally resistant parasite strains; and intense adverse effects, often leading to treatment cessation.<sup>2,3</sup>

Phosphodiesterases (PDEs) are a group of highly conserved hydrolases that control the intracellular levels of cyclic nucleotides by hydrolysing the second messengers cAMP and/or cGMP, both in mammalian hosts<sup>4</sup> and parasites.<sup>5</sup> The high level of conservation between PDEs from mammals and trypanosomatids could be advantageous<sup>6,7</sup> as PDEs are already a pharmacological target in humans and some of the parasite homologues are essential.<sup>8</sup> In kinetoplastids, four families of class I PDEs (A–D) have been identified<sup>9,10</sup> and the enzymes are involved in essential functions including cell division, osmoregulation and virulence.<sup>10–14</sup>

The *Trypanosoma* PDEs were pharmacologically validated as a therapeutic target for *Trypanosoma brucei* using the tetrahydrophthalazinone NPD-001 (previously known as Cpd A).<sup>14</sup> It induced a dose-dependent increase in the cAMP intracellular content in bloodstream forms, leading to immediate inhibition of proliferation and a complete loss of viability 72 h after drug exposure,<sup>14</sup> similarly to the phenotype observed upon RNAi knockdown of the TbrPDE-B1 and TbrPDE-B2 genes.<sup>10</sup> The tetrahydrophthalazinones have now been established as a promising antiparasitic scaffold. Novel NPD-001 analogues have been evaluated for effects on parasite viability and for their inhibition of TbrPDE-B1. Small structural variations were shown to lead to drastic potency differences against human and *T. brucei* PDE subtypes, likely due to the presence of the parasite-specific P-pocket next to the conserved substrate binding site.<sup>15–18</sup>

The aim of this study was to evaluate the activity and selectivity of NPD-001 and a further 11 phthalazinone derivatives against *T. cruzi*, using a well-established screening flow chart based on the Target Product Profile for Chagas' disease,<sup>19</sup> followed by an *in vitro* exploration of combination therapy with benznidazole. Effects of selected inhibitors on ultrastructure and cAMP levels were determined and the mRNA levels of PDEs in amastigotes, epimastigotes and bloodstream forms were evaluated. The findings support the targeting of PDEs for drug discovery against Chagas' disease.

## Materials and methods

### PDE inhibitors and drugs

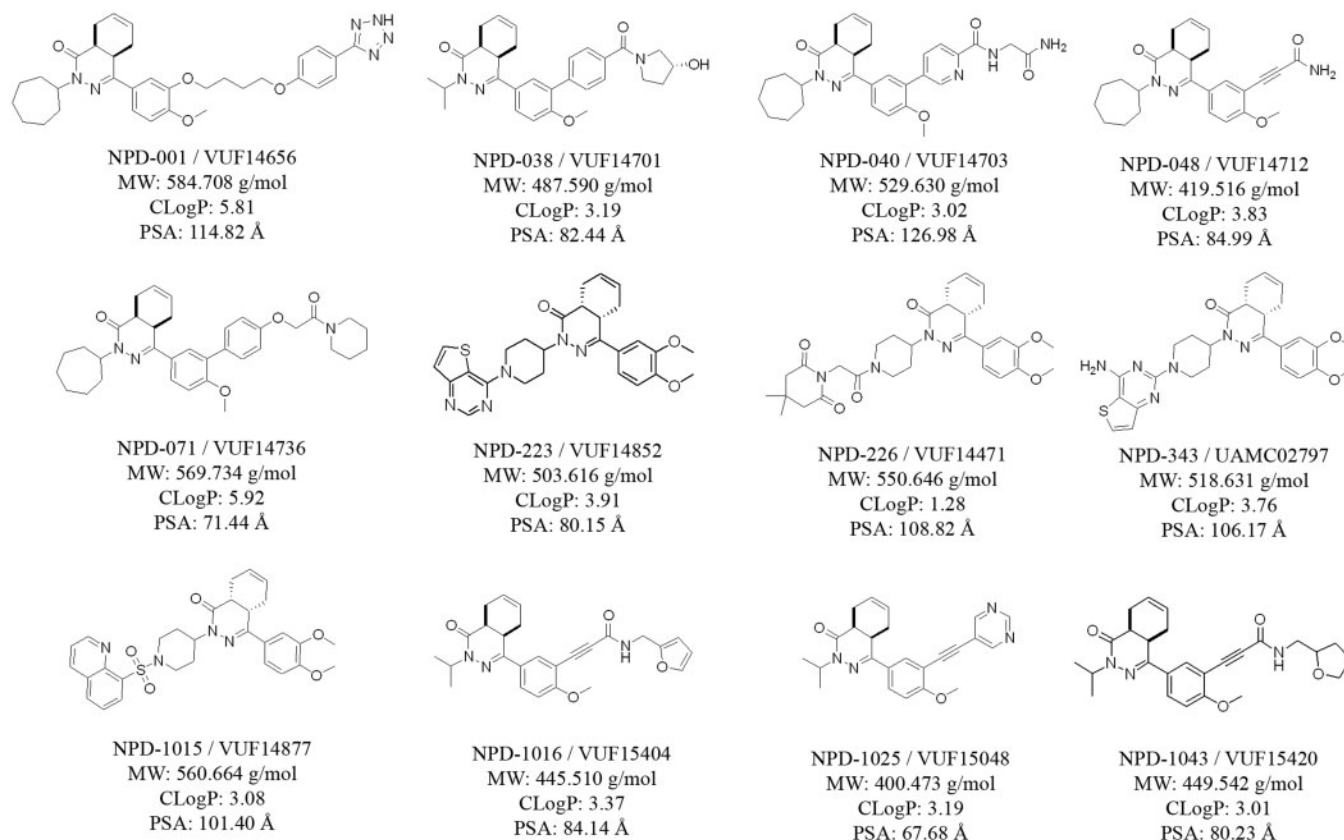
Stock solutions (10 or 20 mM) of 12 compounds (Figure 1) were prepared in DMSO (1% maximum final concentration). Benznidazole (Laboratório Farmacêutico do Estado de Pernambuco, Brazil) was used as a reference drug.<sup>20</sup> Identity and purity information is provided for most active compounds on bloodstream trypomastigotes (BTs) and/or intracellular forms, i.e. NPD-001, NPD-040, NPD-048, NPD-223, NPD-1015 and NPD-1016 (Table S1, available as Supplementary data at JAC Online).

### Mammalian cells

Primary cardiac cell (CC) and L929 fibroblast lineage cultures were obtained as reported previously.<sup>21,22</sup>

### Parasites

BTs of Y [Discrete Typing Unit (DTU) II] and Colombiana (DTU I) strains of *T. cruzi* were obtained by cardiac puncture of infected Swiss Webster mice at peak parasitaemia.<sup>21,23</sup> Trypomastigotes of the Tulahuen strain (DTU VI) expressing the *Escherichia coli*  $\beta$ -galactosidase gene were maintained as reported previously.<sup>20</sup> Extracellular amastigotes of the Y strain were obtained from the supernatant of infected cultures of CCs (50:1, parasite: host cell) as reported previously.<sup>24</sup> Epimastigotes of Y and Colombiana strains were cultivated using a routine method.<sup>23</sup>



**Figure 1.** Chemical structure of the phthalazinones used in this study. MW: molecular weight; CLogP, calculated octanol–water partition coefficient; PSA, polar surface area.

## Cytotoxicity assays

Non-infected CC and L929 cultures were incubated at 37°C for 24–96 h with increasing concentrations of each compound (up to 200 µM) diluted in RPMI. Cellular viability was determined by the PrestoBlue (CC) and AlamarBlue (L929) tests and the morphology and spontaneous contractibility of CCs were evaluated by light microscopy. The results were expressed as the difference in reduction between treated and non-treated cells. LC<sub>50</sub> values (concentration reducing the labelling related to cellular viability by 50%) were determined by non-linear regression (sigmoid curve).<sup>20</sup>

## Trypanocidal activity

BTs of the Y strain ( $5 \times 10^6$  parasites/mL) were incubated for 2 and 24 h at 37°C in RPMI in the presence or absence of 1:3 serial dilutions (six points) of the compounds (0–200 µM) for direct quantification of live parasites by light microscopy, for calculation of the EC<sub>50</sub> (compound concentration that reduces the number of parasites by 50%).<sup>20</sup> For the analyses of compound effects on intracellular forms, *T. cruzi*-infected L929 cultures (Tulahuen strain) were incubated for 96 h at 37°C with each compound at non-toxic concentrations (six points) followed by calculation of EC<sub>50</sub>, as reported.<sup>23</sup> For extracellular amastigotes of the Y strain, the parasites were incubated ( $5 \times 10^6$ /mL) with increasing concentrations of the selected compounds for 24–96 h and the EC<sub>50</sub>s determined through direct quantification by light microscopy.<sup>24</sup> The selectivity index (SI) is the ratio between LC<sub>50</sub> (toxicity for mammalian cells) and the EC<sub>50</sub> (activity against the parasite). The pEC<sub>50</sub> represents the negative log of the EC<sub>50</sub> value  $\{-\log[EC_{50} (M)]\}$ .

## In vitro combination therapy

Drug interactions were investigated in the Tulahuen strain of *T. cruzi* using a fixed-ratio method<sup>25</sup> by combining the selected compound with benznidazole, following 96 h of incubation at 37°C. Predetermined EC<sub>50</sub> values were used to determine the top concentrations of the individual ratios ensuring that the EC<sub>50</sub> fell in the midpoint of a 7-point 2-fold dilution series. The fixed ratios of 5:0, 4:1, 3:2, 2:3, 1:4 and 0:5 were used, as previously reported.<sup>26</sup>

## Determination of FIC index (FICI), classification of interaction and isobologram construction

FICIs and the sum of the FICIs (ΣFICI) were calculated using the following formula: FICI of tested compound = EC<sub>50</sub> of tested compound in combination/EC<sub>50</sub> of tested compound alone. The same equation was applied to benznidazole and ΣFICI = FICI (inhibitor) + FICI (benznidazole). An overall mean ΣFICI was calculated for each combination and used to classify the nature of the interaction, as follows: 'synergy' for xΣFICI ≤ 0.5, 'antagonism' for xΣFICI > 4.0 and 'no interaction' or 'additive' for xΣFICI > 0.5–4.0 (xΣFICI indicates the overall mean ΣFICI).<sup>27</sup> Isobolograms were constructed by plotting the EC<sub>50</sub> of NPD-040 against the EC<sub>50</sub> of benznidazole.<sup>26</sup>

## Cloning of TcrPDEs

Genomic DNA was extracted from Y and Colombiana strain parasites. To ascertain the correct start and stop sequences for the five genes, PCRs first amplified from the untranslated regions flanking each gene, either to primers within the genes (PDEs B1, B2 and C) or across the coding region (PDEs A and D), using a proofreading polymerase (Phusion; NEB, Hitchin, UK; primers in Table S2). Amplified fragments were ligated into the pGEM-T Easy subcloning vector (Promega, Southampton, UK) and Sanger sequenced (Source BioScience). This sequence information was used to design primers to amplify the complete coding sequence for each PDE from both Y strain and Colombiana genomic DNA, again using Phusion polymerase (Table S2), followed by ligation into pGEM-T Easy and Sanger sequencing. All sequences were submitted to GenBank (Table S3).

## qRT-PCR

Samples of *T. cruzi* Y strain epimastigotes, BTs and amastigotes collected from the supernatant of infected CC cultures were stabilized in RNeasy lysis buffer (Thermo Fisher). RNA was extracted using a Macherey-Nagel NucleoSpin RNA extraction kit and cDNA produced using Superscript III reverse transcriptase (Thermo Fisher). The expression profile of each of the five *T. cruzi* PDEs was generated from the three-stage cDNAs. GoTaq qPCR master mix (Promega) was used to amplify fragments, with an Applied Biosystems 7500 Real Time PCR System (primers in Table S2). The expression levels were calculated relative to the *T. cruzi* housekeeping gene *GAPDH* as a control (primers from Silber et al.<sup>28</sup>).

## Electron microscopy

BTs from the Y strain ( $5 \times 10^6$  parasites/mL) were treated with the selected compound for 2 h at the EC<sub>50</sub> concentration. The parasites were fixed with 2.5% glutaraldehyde and processed as routine for transmission (TEM) and scanning (SEM) electron microscopy.<sup>29</sup>

## cAMP measurement

Extracellular amastigotes ( $2 \times 10^7$  parasites/mL) or BT forms of Y strain ( $1.5 \times 10^7$  parasites/mL) were treated with NPD-001 or NPD-040 at a concentration of  $2 \times EC_{50}$  for 2.5 h at 37°C, processed and analysed using a cAMP ELISA kit (Cayman Chemicals, MI, USA).<sup>14</sup>

## Statistical analysis

Statistical analysis was performed using Student's *t*-test with the level of significance set at  $P \leq 0.05$ .

## Ethics

All animal procedures performed at Fundação Oswaldo Cruz (FIOCRUZ) were carried out in accordance with the guidelines established by the Committee of Ethics for the Use of Animals (CEUA LW16/14).

## Results

### Phenotypic screening of potential PDE inhibitors against relevant *T. cruzi* forms

Compounds assayed for activity against intracellular forms of the β-galactosidase-transfected Tulahuen strain (DTU VI) showed that 4 out of 12 inhibitors exhibited EC<sub>50</sub> values up to 10 µM, established as the threshold for hit compounds for *T. cruzi*.<sup>30</sup> However, none of them presented better activity or superior selectivity than benznidazole (EC<sub>50</sub> =  $2.7 \pm 0.4$  µM, SI = 51) in this system (Table 1). To evaluate the effect against another very relevant parasite form and DTU for mammalian infection, additional phenotypic approaches were conducted using BTs from the Y strain (DTU II). In these assays, three compounds were at least as active as benznidazole: NPD-001 (PDE inhibitor reference compound), NPD-040 and NPD-048. The latter was twice as potent as benznidazole, with EC<sub>50</sub> values of  $6.25 \pm 0.68$  µM and  $12.9 \pm 1.9$  µM, respectively, after 24 h of incubation at 37°C; the NPD-040 EC<sub>50</sub> was  $12.1 \pm 0.8$  µM (Table 2). For NPD-223, NPD-1015 and NPD-1016, major differences in potencies were observed depending on the parasite form, with EC<sub>50</sub> values from 5.1–9.4 µM or from 54 up to >200 µM for intracellular (Tulahuen strain) and BT (Y strain) forms, respectively (Tables 1 and 2).



**Table 1.** Activity and selectivity of studied molecules against intracellular amastigotes from  $\beta$ -galactosidase-transfected Tulahuen strain of *T. cruzi* incubated for 96 h at 37°C

Corp ID	Molecule name	EC <sub>50</sub> $\mu$ M (mean $\pm$ SD)	pEC <sub>50</sub>	SI against L929 cultures
VUF14656	NPD-001	>10	<5	<3.5
VUF14701	NPD-038	>10	<5	ND
VUF14703	NPD-040	7.8 $\pm$ 1.7	5.11	3.6
VUF14712	NPD-048	>10	<5	ND
VUF14736	NPD-071	>10	<5	ND
VUF14852	NPD-223	9.4 $\pm$ 0.9	5.02	3
VUF14471	NPD-226	>10	<5	ND
UAMC02797	NPD-343	>10	<5	ND
VUF14877	NPD-1015	6.2 $\pm$ 2.4	5.21	>32
VUF15404	NPD-1016	5.1 $\pm$ 2.3	5.29	>39
VUF15048	NPD-1025	>10	<5	ND
VUF15420	NPD-1043	>10	<5	ND
Benznidazole	NPD-1265	2.7 $\pm$ 0.4	5.57	51

ND, not defined; Corp ID, corporate identification number.

**Table 2.** *In vitro* activity and selectivity of the studied molecules against BTs from the Y strain of *T. cruzi* incubated for 24 h at 37°C

Corp ID	Molecule name	EC <sub>50</sub> $\mu$ M (mean $\pm$ SD)	pEC <sub>50</sub>	SI
VUF14656	NPD-001	9.12 $\pm$ 3.37	5.04	5.6
VUF14701	NPD-038	30.29 $\pm$ 3.41	4.52	>6.6
VUF14703	NPD-040	12.1 $\pm$ 0.8	4.92	>8.3
VUF14712	NPD-048	6.25 $\pm$ 0.68	5.20	>32
VUF14736	NPD-071	>200	<3.70	ND
VUF14852	NPD-223	54.47 $\pm$ 48.91	4.26	>1.8
VUF14471	NPD-226	178 $\pm$ 38	3.75	>1.12
UAMC02797	NPD-343	>200	<3.70	ND
VUF14877	NPD-1015	>200	<3.70	ND
VUF15404	NPD-1016	125 $\pm$ 0	3.90	>1.6
VUF15048	NPD-1025	70.2 $\pm$ 9.48	4.15	>2.8
VUF15420	NPD-1043	56.7 $\pm$ 4.10	4.25	>3.5
Benznidazole	NPD-1265	12.9 $\pm$ 1.9	4.3	77

ND, not defined; Corp ID, corporate identification number.

NPD-040 (one of the most active against both parasite forms and strains) and benznidazole were further assayed against free amastigotes of the Y strain obtained from the supernatant of *T. cruzi*-infected CC cultures after host cell rupture induced by superinfection. Benznidazole and NPD-040 presented a time-dependent effect, with the latter acting faster, reaching EC<sub>50</sub> values diminishing from 7.7 to 4  $\mu$ M after 24–96 h of PDE inhibitor exposure (Figure 2).

### Exploration of combinations with benznidazole

As combinatory approaches represent an interesting strategy of improving efficacy by tackling distinct targets with multiple drugs, a fixed-ratio combinatory assay was performed using NPD-040

and benznidazole, due to its consistent anti-*T. cruzi* behaviour. Using the classification proposed by Odds,<sup>27</sup> the x $\Sigma$ FICI values for the benznidazole and NPD-040 combination revealed a profile close to synergy against intracellular amastigotes (x $\Sigma$ FICI=0.58) but additive (no interaction) against BT forms (x $\Sigma$ FICI=1.27) (Figure 3).

### Ultrastructural changes after treatment with NPD-040

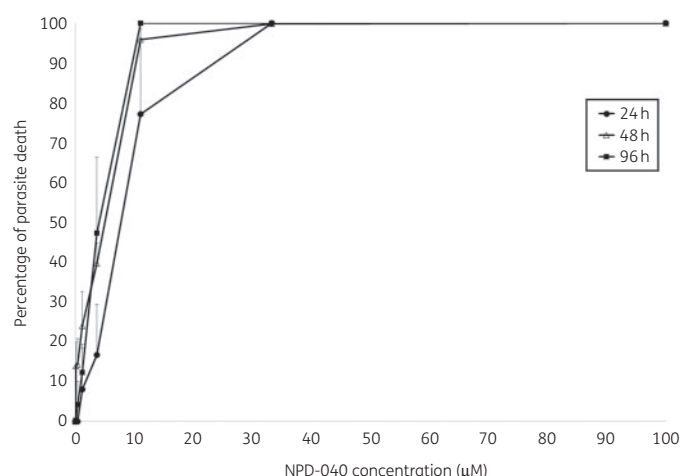
In order to visualize the early ultrastructural damage triggered by NPD-040, electron microscopy analyses were conducted using BTs of the Y strain (treated for 2 h using the corresponding EC<sub>50</sub> of 24 h). As seen in Figure 4(a and b), SEM did not reveal major morphological alterations to the morphology as compared with untreated BTs, except for a swollen aspect at the posterior end of the treated parasite. TEM demonstrated early and common insults related to flagellar pocket dilatation, disruption of Golgi apparatus, myelin figures and endoplasmic reticulum profiles surrounding cytoplasmic organelles (Figure 4c–g).

### Target validation by measurement of cAMP

In order to validate that NPD-001 and NPD-040 targeted *T. cruzi* PDEs, their effects upon the cAMP content of BTs and CC-released amastigotes were evaluated using concentrations of 2 $\times$  the EC<sub>50</sub> value and 2.5 h of incubation for each compound. Both compounds induced highly significant increases in the intracellular cAMP levels of BTs ( $P < 0.001$ ; Figure 5a). Similar levels ( $P < 0.001$ ) were observed at longer incubation times (5 h) and higher inhibitor concentrations (5 $\times$ ) (data not shown). Both inhibitors also stimulated the release of cAMP by BTs into the extracellular medium, leading to a detectable rise of the cAMP concentration within 2.5 h ( $P < 0.001$ ; Figure 5b). For amastigotes, the increase of intracellular cAMP was somewhat more modest but still significant ( $P < 0.001$ ; Figure 5c) and there was no significant increase in the supernatant medium after 2.5 h (Figure 5d). Thus, BTs and extracellular amastigotes displayed similar responses after 2.5 h of incubation with PDE inhibitors at 2 $\times$  EC<sub>50</sub>, with the reduced magnitude of the observed response in amastigotes possibly attributable to the higher rate of cAMP export found in these forms, as the medium cAMP level was 6.7-fold higher for amastigotes than for BTs (Figure 5b and d).

### Expression analysis of *T. cruzi* PDEs

As part of the interpretation of the results with PDE inhibitors, we investigated the expression levels of all *T. cruzi* PDEs in the relevant parasite stages. The confirmed sequences, which deviated mildly from the reference genome sequence (CL Brener strain) (Figure S1), were all deposited at GenBank (Table S3). qRT-PCR analysis was performed with cDNA from BTs, amastigotes and epimastigotes (Figure 6). The results were normalized in each parasite form according to their PDE-C level expression. The findings show that all PDEs are expressed in all three parasite forms. In BTs and epimastigotes, the expression level of PDE-A was clearly below that of the other PDEs. In amastigotes, the expression of PDE-A was very similar to PDEs B1, C and D, with PDE-B2 expressed at almost twice the level of the other enzymes. In epimastigotes, PDE-D expression was the highest, at  $\sim 200\%$  of PDE-C (Figure 6).



**Figure 2.** Kinetic profile of NPD-040 activity upon extracellular amastigotes of the Y strain, following 24, 48 and 96 h of treatment.

## Discussion

Numerous PDE inhibitors are in current clinical use and some of these are being pursued as antiparasitic agents through repurposing.<sup>31–33</sup> A sequence alignment of mammalian class I PDEs with *T. brucei* PDE-B1 and PDE-B2 first revealed ~30%–40% identity<sup>34</sup> and led to genetic and pharmacological studies that established these enzymes as promising drug targets, with the phthalazinone NPD-001 and its analogues as potent lead compounds.<sup>10,13,14,17</sup> In *T. cruzi*, PDE-C was reported as a pharmacologically validated drug target<sup>11</sup> and we therefore investigated whether the phthalazinones also display activity against this trypanosome species.

Phthalazinone derivatives have been studied for decades and are known for their anti-inflammatory,<sup>35,36</sup> anti-aggregating<sup>37,38</sup> and antihypertensive<sup>39,40</sup> properties. Actually, all these features are highly connected to their ability to inhibit different PDE isoforms, interfering with a large number of biological pathways by increasing cellular cyclic nucleotide levels.<sup>41</sup> In kinetoplastids, cell cycle regulation<sup>6</sup>, osmoregulation<sup>13</sup> and cytokinesis events<sup>10,14,42</sup> are under control of cyclic nucleotide signalling. Moreover, cAMP is implicated in immune suppression by the infecting parasite.<sup>43</sup>

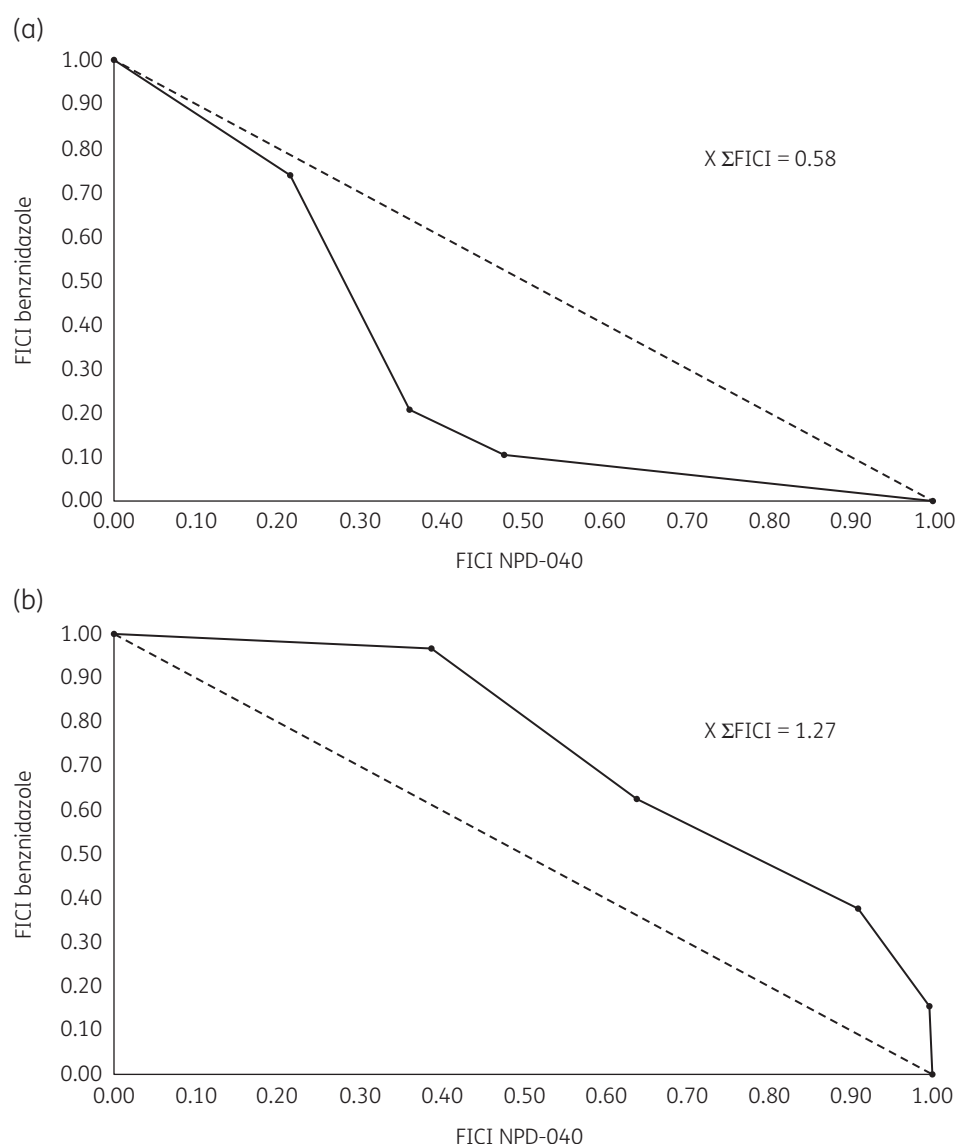
Presently, we report the evaluation of NPD-001 and 11 structurally related phthalazinone derivatives against *T. cruzi*. Four of the inhibitors were active against intracellular amastigotes ( $EC_{50} \leq 10 \mu M$ ), with two of them, NPD-1015 and NPD-1016, fulfilling the primary criteria of a hit compound for Chagas' disease ( $SI \geq 10$ ).<sup>29</sup> However, the activity and selectivity of both derivatives were lost when screened against BTs (the other relevant parasite form for *T. cruzi* infection), exhibiting  $EC_{50}$  values in excess of 100  $\mu M$ .

The lack of corresponding biological activity while assaying both bloodstream and intracellular forms was also noticed with other studied phthalazinones such as NPD-223 and some possible explanations could be raised, including the use of different parasite strains (Y for BT and Tulahuen for intracellular forms). Another possibility is the potential impact of PDE inhibitors on the host cell physiology, indirectly affecting parasite survival. These issues could result in different potency outcomes for assays with intracellular and extracellular forms, for reasons of host cell physiology rather than direct inhibition of parasite PDEs. To address these issues,

amastigotes naturally released by CC cultures were employed, but now using the Y strain (the same as previously tested on BTs) and incubations from 24 up to 96 h. Although benznidazole displayed a remarkably time-dependent trypanocidal effect, the phthalazinone NPD-040 sustained a comparatively equivalent action during the entire incubation period, with the  $EC_{50}$  values between 4.0 and 7.7  $\mu M$ . These potencies are very similar to those found against intracellular amastigotes (Tulahuen strain) in L929 cells ( $EC_{50} = 7.8 \mu M$ ), which argues against any potential difference related to parasite strain. Additional studies with NPD-001 also showed a similar potency against amastigotes (22 and 20  $\mu M$  for the Tulahuen and Y strain, respectively) regardless of the parasite DTU and parasite localization (intracellularly or in a cell-free system), which disproves the possibility that these phthalazinones act indirectly on the amastigotes by impacting the host cell metabolism. However, other aspects may be considered including the metabolic differences of the highly proliferative amastigotes and the non-dividing but infective BTs, which could influence their different susceptibility to drugs manipulating cAMP.<sup>44</sup> Another distinct possibility would be the differential expression of individual PDEs in the various lifecycle forms and we did find some evidence for this, such as with PDE-A and PDE-B2 being somewhat higher expressed in the amastigote stage than in trypomastigotes. Hard evidence of which PDE is being targeted will require systematic assessment of the essentiality of each PDE in the various life cycle stages and inhibitor studies on each separate enzyme.

Some differences in susceptibility according to parasite form may be attributable to the ability of the host cells to take up or extrude the compound. For instance, NPD-048 displayed higher  $EC_{50}$  values for intracellular amastigotes of *T. cruzi*, regardless of parasite strain (12 and 18  $\mu M$ ), than against extracellular parasites (BTs and extracellular amastigotes: 6.2  $\mu M$  for both), suggesting that penetration of the mammalian host cell may be limiting its efficacy.

A further factor in differential activity against amastigotes and BTs could be the observation that amastigotes seem to release cAMP much faster and in higher levels than BTs, which might make them less vulnerable to PDE inhibition. In both BTs and amastigotes, the levels of cAMP in intracellular contents increased rapidly in the presence of PDE inhibitors and were highly significantly elevated at the 2.5 h point, reminiscent of a recent study on *T. cruzi* that reported a high and very rapid (2 h) increase in cAMP levels under the nutritional stress conditions that trigger metacyclogenesis.<sup>45</sup> The genome of trypanosomatids encodes five different isoforms of PDEs<sup>33,46</sup> and we report the cloning and sequencing of all the PDE genes for the Y and Colombiana strains, and their relative level of expression in amastigotes, BTs and epimastigotes. The cAMP measurements clearly show that, like *T. brucei*,<sup>14</sup> *T. cruzi* regulates its intracellular cAMP level in two ways: (i) degradation by PDEs; and (ii) extrusion to the extracellular environment, which in mammalian cells is mediated by ATP-binding cassette (ABC) transporters.<sup>47,48</sup> The cAMP concentration in the extracellular medium of untreated amastigote suspensions was ~6.7-fold higher than in the untreated trypomastigote suspension, reflecting a much higher rate of natural basal efflux by untreated amastigotes and this allows the untreated amastigotes to maintain the same steady-state intracellular cAMP concentration as the untreated trypomastigotes (22.6±1.1 versus 23.3±1.7  $\mu M$ , values normalized according to each cellular volume;  $P > 0.05$ ; Figure 5). As the overall PDE expression levels in amastigotes and trypomastigotes only



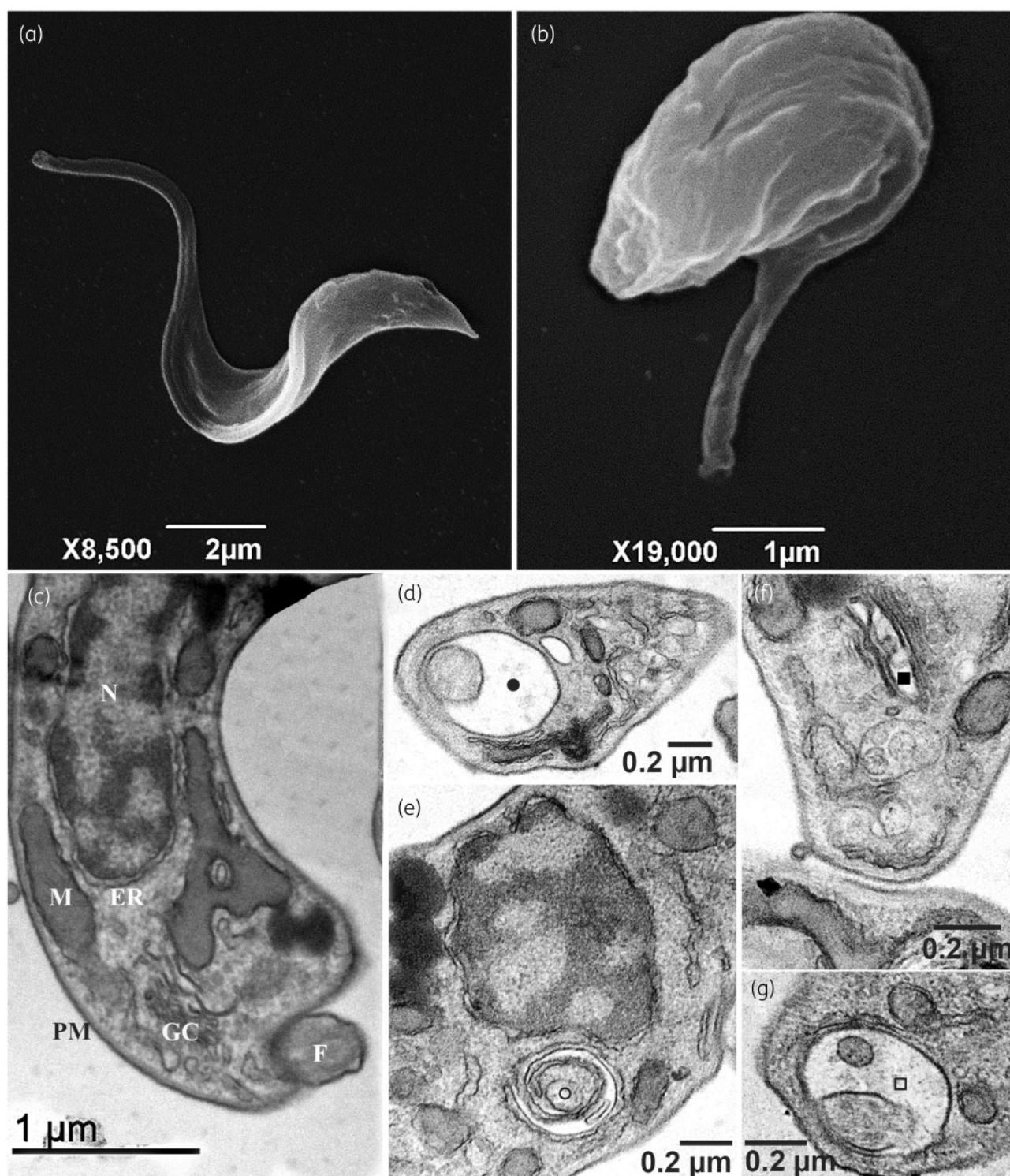
**Figure 3.** *In vitro* compound interactions of NPD-040 and benznidazole upon *T. cruzi*, using a fixed-ratio method on intracellular forms of Tulahuen strain (a) and BTs of the Y strain (b). Dose-response experiments were performed with NPD:BZ at 5:0, 4:1, 3:2, 2:3, 1:4 and 0:5 fixed ratios, using the predetermined EC<sub>50</sub> values to establish the top concentrations.

vary modestly in the various isoforms, it appears that cAMP production, signalling and efflux are likely much more active in amastigotes. Thus, for the two inhibitors tested here, cAMP accumulation in BTs reached substantially higher concentrations than in amastigotes, due to their lower capacity for efflux, as evidenced by the much lower extracellular levels in the trypomastigote suspension.

*T. cruzi* PDEs have previously been implicated in osmotic control.<sup>49</sup> As initially observed by light microscopy (data not shown), BT forms exposed to NPD-040 display a swollen morphological aspect, which was clearly observed by SEM, even after short periods of compound incubation, and may reflect an altered osmotic balance. The ultrastructural analysis performed by TEM showed that this phthalazinone rapidly causes damage to the Golgi and the

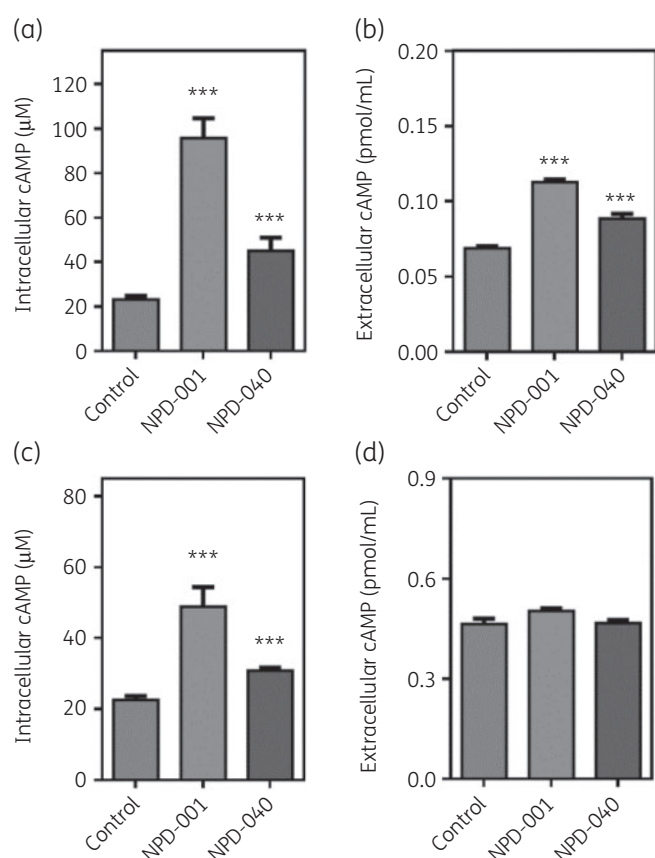
flagellar pocket and induces membranous profiles surrounding intracellular organelles that resemble autophagy, as already reported for other trypanocidal agents,<sup>50,51</sup> and this merits further investigation.

Combination therapy is a valuable tool to improve treatment efficacy by reducing dose levels and toxicity and preventing the development of drug resistance.<sup>52,53</sup> We thus investigated the merits of a combination of benznidazole and NPD-040. Although for BTs the xΣFICI value of 1.27 indicated no interaction (additive profile), for intracellular forms a close-to-synergistic response was obtained (xΣFICI = 0.58). The distinct profile could be related to differences of metabolic states of replicating amastigotes and non-dividing trypomastigotes, respectively. Considering that in *T. brucei* the effect of high cellular cAMP is to disrupt cell division

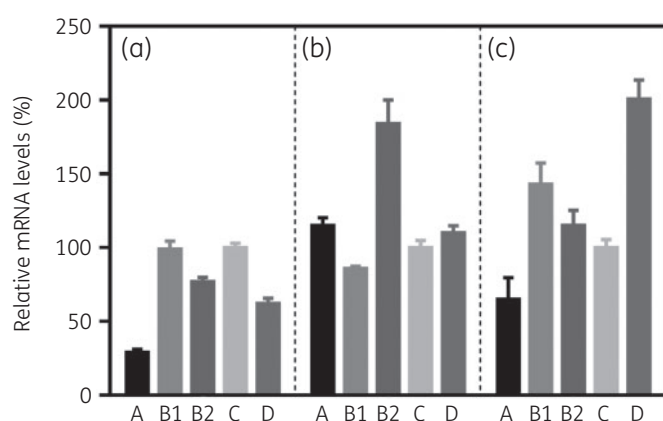


**Figure 4.** Electron microscopy of a BT of *T. cruzi* (Y strain), untreated (a and c) and exposed for 2 h to NPD-040 (b and d–g) and evaluated by SEM (a and b) and TEM (c–g). Parasites treated with NPD-040 displayed flagellar pocket dilatation (filled circle), disruption of Golgi apparatus (filled square), myelin figures (open circle) and endoplasmic reticulum profiles (open square) surrounding cytoplasmic organelles. PM, plasma membrane; GC, Golgi complex; ER, endoplasmic reticulum; F, flagellum; M, mitochondria; N, nuclei.





**Figure 5.** cAMP concentrations for BTs (a and b) and extracellular amastigotes (c and d) treated with NPD-001 and NPD-040. Results of cell content samples are displayed in graphs (a) and (c), while values for extracellular medium samples are shown in graphs (b) and (d). \*\*\* $P < 0.001$  by Student's unpaired  $t$ -test.



**Figure 6.** Expression of all PDEs analysed with cDNA from BTs (a), amastigotes from myocardial cells (b) and insect-stage epimastigotes (c). All normalized to the level of expression of the housekeeping gene *GAPDH* and with the expression level of PDE-C set at 100% in each line.

through the inhibition of cytokinesis,<sup>14</sup> the lesser effect of (some) PDE inhibitors on the non-dividing trypomastigotes may not be that surprising.

Human PDEs have a long history as drug targets and several inhibitors already on the market are used for a wide spectrum of clinical conditions.<sup>54</sup> Hence, extensive experience in the clinical development of PDE inhibitors is available and this may help to develop parasite-selective inhibitors. Issues related to the specificity can be overcome through medicinal chemistry guided by crystal structures.<sup>8,15</sup> Knowledge transfer from the field of human PDE inhibitors, together with a collaborative, multidisciplinary approach to drug discovery, provides new opportunities to couple the curiosity-driven research culture in academia with the stringent preclinical practices used in industry.<sup>55</sup> In this way, the cumulative knowledge and skill of the private and academic sectors may be employed for drug discovery programmes focused on neglected pathologies like Chagas' disease, which has millions of patients waiting for safer and more effective therapies.

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## Transparency declarations

None to declare.

## Supplementary data

Tables S1-S3 and Figure S1 are available as [Supplementary data](#) at JAC Online.

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